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*Published in:*
Biochimica et Biophysica Acta

*DOI:*
10.1016/S0005-2728(02)00252-9



*Publication date:*
2002

ELSEVIER

Biochimica et Biophysica Acta 1555 (2002) 44–47

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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# Peroxisomes: surprisingly versatile organelles

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Received 17 May 2002; accepted 4 June 2002

**Abstract**

Peroxisome development is a dynamic process that is not yet completely understood. We use the methylotrophic yeast *Hansenula polymorpha* as model in our studies on peroxisome homeostasis. Cells of this species may contain different types of peroxisomes that differ in protein composition and capacity to incorporate matrix proteins. This protein import machinery is highly flexible and can accommodate unfolded and complex folded proteins.


*Keywords:* Peroxisome; Organelle; *Hansenula polymorpha*

## 1. Introduction

Peroxisomes are ubiquitous cell organelles that strongly vary in abundance and function [1,2]. In fungi, their function ranges from a crucial role in penicillin biosynthesis to a function in nematode capturing by certain nematophagous fungi [3] or sealing septal pores in hyphae [4]. The reason as to why these organelles have gained such different functions in various organisms is fully unclear. Characteristic for peroxisomes is their extremely high matrix/membrane protein ratio. The low abundance of large integral membrane proteins in peroxisomes of the yeast *Hansenula polymorpha* is convincingly illustrated by the typical smooth fractures faces of the peroxisomal membrane in freeze etch replicas [5].

In recent years, much has been learned on the protein components that are essential for peroxisome biogenesis. Many of these proteins, termed peroxins, have been identified by functional complementation of yeast mutants, deficient in peroxisome biogenesis (*pex* mutants). Various components involved in matrix protein import, membrane biogenesis, organelle fission and movement have been identified and the first details on their molecular functions are emerging. However, several controversial topics have been discerned, the solution of which is essential for the progress in the field, and which is topic of discussion in this paper.

## 2. Peroxisome development

In normal wild-type cells, peroxisomes multiply by division. The first clear cut examples of peroxisome division as a mode to supply developing cells (buds) with new organelles have been described in yeast [6]. Later, Lazarow and Fujiki [7] provided biochemical evidence for this mode of peroxisome proliferation. Remarkably, morphological evidence for peroxisome fusion events, comparable to for instance vacuole and mitochondrial compartments, has not been reported yet. In addition, fusion of mature peroxisomes seems unlikely as huge organelles, resulting from this, have never been observed in yeast cells. The only example known in the yeast *H. polymorpha* resulted from the overexpession of alcohol oxidase (AO) protein in a mutant strain of this yeast species [8]. Large peroxisomes have also been observed in other yeast species defective in the peroxin Pex11p [9,10]. Most likely, this is due to a failure in fusion instead of being a result of fusion. Normally, the organelles are quite comparable of size, giving support to the notion that growth by matrix protein import may cease at a certain stage of development or alternately, that the putative removal of waste proteins and new protein uptake are at a state of equilibrium. However, we have obtained evidence that peroxisomes in methylotrophic yeast are only temporally

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0005-2728/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0005-2728(02)00252-9

matrix protein import competent [11] and showed that import was in fact confined to few small organelles that were present in the cells. This implies that the cells contain different classes of peroxisomes, namely mature organelles that apparently have lost the capacity to incorporate matrix proteins (and thus can be considered ''enzyme bags'') and a minor number of small organelles that can grow and multiply. However, several questions remain that require an unequivocal answer:

(i) Is the above mode of peroxisome development conserved or only valid for methylotrophic yeast species?
(ii) Is the capacity to import matrix proteins donated from one organelle to another [12] or are organelles continuously (one after the other) formed from specific organelles or a peroxisome reticulum?
(iii) What is the molecular basis for the discrimination between import competent and incompetent organelles?
(iv) Do alternative modes of peroxisome biogenesis exist?

## 3. Alternative modes of peroxisome formation

Titorenko et al. [2,13,14] were the first to provide clear-cut evidence that peroxisome development in *Y. lipolytica* involves membrane fusion events. These workers demonstrated that peroxisomes develop by a multistep process that initiates with the formation of preperoxisomal vesicles that arise from a subdomain of the ER. These structures harbour distinct subsets of membrane proteins as well as components of COPII vesicles and transform into early peroxisomal precursors, designated P1 and P2, as a result of the uptake of additional membrane proteins and release of the COPII elements. P1 and P2 peroxisomes are competent to incorporate distinct sets of matrix proteins and fuse in a Pex1p/Pex6p dependent way to generate P3 peroxisomes that develop into mature peroxisomes by a multistep assembly pathway via P4 and P5 peroxisomes. Also in peroxisome-deficient human fibroblasts, evidence was obtained for a multistep peroxisome assembly pathway that occurred upon reintroduction of *PEX16* in cells of a Pex16p-defective cell line [15]. In this system, Pex16p is incorporated in a preperoxisome, followed by the insertion of other PMPs that enables subsequent matrix protein import. These preperoxisomes are autonomous structures that do not arise from the ER and assemble into nascent peroxisomes independent of COP proteins [16] or the ER translocon [17].

We showed that in *H. polymorpha pex3* cells the endomembrane system may serve as template for the formation of new peroxisomes [18]. Upon synthesis of the initial 50 amino acids of Pex3p ($N_{50}$.Pex3p) in *H. polymorpha pex3*, various vesicles were formed that arose from the nuclear envelope. These vesicles showed peroxisomal characteristics and contained, apart from $N_{50}$.Pex3p, other peroxisomal membrane proteins. Upon subsequent synthesis of full-length Pex3p, a portion of these vesicles developed into normal peroxisomal peroxisomes.

In concept, the models proposed for *Y. lipolytica* and *H. polymorpha pex3* display comparable properties as they suggest that (re-)introduction of peroxisomes initiates at the endomembrane system. However, the *Y. lipolytica* model proposes that this pathway occurs in WT cells upon induction of peroxisome formation. The reintroduction models explain how peroxisomes assemble in cells that were fully devoid of peroxisomes due to genetic defects. It is unclear yet whether such a mechanism is also operative in cells that grow normally at peroxisome-inducing conditions. In *H. polymorpha*, the 'normal' pathway of growth and division became operative upon prolonged cultivation in cells in which peroxisome formation initially was started by $N_{50}$.Pex3p-induced vesicles. Thus, in this organism, the above mechanism of peroxisome recovery may represent a rescue mechanism that becomes functional in case peroxisomes are lost, e.g. due to failure in inheritance.

## 4. Matrix protein import

The matrix protein import machinery is remarkably conserved in low and higher eukaryotes. For these proteins, two peroxisomal targeting signals (PTS1 and PTS2) are detected that are recognized by the cytosolic receptors Pex5p and Pex7p, respectively. Both receptors bind their cargo proteins in the cytosol and guide them to a docking site at the peroxisomal membrane. Dammai and Subramani [19] recently presented evidence that human Pex5p in fact is a cycling receptor, which translocates across the peroxisomal membrane bound to a PTS1-cargo protein and, following release of its cargo, cycles back to the cytosol. This so-called ''extended shuttle model'' was first proposed for the yeast *H. polymorpha* [12,20] and in human cell lines [21]. This finding has the major implication that (i) a protein export machinery must exist for Pex5p and (ii) in case Pex5p dissociates from the inner surface of the membrane, a Pex5p sorting machinery exists in the organellar matrix. Both aspects are yet completely unresolved. It is also not known whether import and export require separate machineries or use one and the same. In this context, it is tempting to speculate that one of the peroxins proposed to function in docking (e.g. Pex13p and Pex14p) in fact may function in Pex5p export. Pex14p may represent a plausible candidate as it is not essential for matrix protein import [22]. Blocking Pex5p recycling would slow down matrix protein import to a very low level, as in *pex14* strains. This is in line with the phenotype of the *H. polymorpha pex4* mutant, which contains peroxisomal remnants that contain AO protein [23]. Pex14p is also essential for selective peroxisome degradation in *H. polymorpha*. Bellu et al. [24] showed that the information that governs the degradation process is located in the extreme N-terminus of the protein. This means that Pex14p may have multiple functions and act as a molecular

switch that discriminates between two oppositely directed processes namely organelle assembly and selective degradation. Pex14p may also be—part of—the clue that determines the temporal import capacity of peroxisomes because of a change in modification (phosphorylation [25,26]) or in topology of the N-terminus.

The current models on PTS1 protein import generally propose a single import pathway. However, as specific PTS1 proteins (e.g. acyl CoA oxidase [27]) are only imported as oligomers, whereas import of AO is restricted to monomers [28], this pathway may be much more versatile. It has to be considered that the PTS1 pathway may exist as separate, probably overlapping tracks. A plausible explanation for a dynamic PTS1 matrix protein import machinery is that delivery of the Pex5p-cargo complex at the peroxisomal membrane is paralleled by the assembly of a docking complex, followed by association of another subset of peroxins into a dynamic translocation pore. Other complexes may subsequently form that are involved in Pex5p export/recycling. Specific complexes that mediate import of folded and unfolded peptides may contain common elements [29], which explains the extensive network of protein–protein interactions that have been discovered among peroxins involved in peroxisomal protein import.

## 5. Assembly of octameric, FAD-containing AO

It has been demonstrated that several peroxisomal matrix proteins are imported into peroxisomes as folded, oligomeric structures. In this respect, AO of methylotrophic yeasts seems to be an exception on this rule. The import and assembly of *H. polymorpha* AO has been topic of investigation for over 10 years. AO is an oligomeric enzyme that consists of eight identical subunits that each contains an FAD-molecule noncovalently bound. In WT cells, the activity of this enzyme is confined to the peroxisomal matrix, and several lines of evidence have lend support to the view that octamerisation occurs inside the organelle upon import of inactive monomers.

Several independent approaches have revealed that octameric AO cannot be transported across the peroxisomal membrane [30,31], whereas other PTS1 proteins are imported as oligomers in *H. polymorpha* peroxisomes [32]. Therefore, most likely, specific proteins are involved in AO import/activation that are not required for other *H. polymorpha* PTS1 proteins. In order to identify such proteins, *H. polymorpha* mutants were isolated that are blocked in AO import and activation. These mutants display a strongly reduced AO activity and, as a consequence, fail to grow on methanol. Complementation analysis of the mutants available so far revealed the presence of 10 different complementation groups [33]. One of these groups has been studied in detail. These mutants are characterised by accumulation of inactive, FAD-lacking monomeric AO in the cytosol while other peroxisomal matrix proteins are normally activated and sorted to peroxisomes. The gene that functionally complemented the AO-assembly defective phenotype in this group of mutants encodes the enzyme pyruvate carboxylase (HpPyc1p). Pyruvate carboxylase is an anapleurotic enzyme, localised in the cytosol, that replenishes the tricarboxylic acid cycle by the synthesis of oxaloacetate. Mutational analyses revealed that not HpPyc1p enzyme activity, but the protein was essential to functionally complement the AO assembly defect in these mutants. Hence, HpPyc1p fulfils a dual role in that, besides its well-characterised metabolic function as anapleurotic enzyme, the protein plays a specific role in the AO sorting and assembly. Because FAD-lacking AO monomers accumulate in the absence of HpPyc1, it is tempting to speculate that HpPyc1p mediates FAD-binding to AO monomers in the cytosol. Previous studies using an *H. polymorpha* riboflavin-deficient mutant (*rif1*) already indicated that FAD-binding is essential to allow efficient import and octamerisation of AO [34,35]. Most likely, newly synthesised AO monomers first bind FAD, mediated by HpPyc1p, followed by binding to the PTS1 receptor Pex5p. Then, FAD-containing monomers bound to Pex5p are taken up by the organelles followed by dissociation of Pex5p. This allows the FAD-containing monomers to oligomerise into the enzymatically active octamers, a process that most likely occurs spontaneously [36].

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